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Evaluation of attractive toxic sugar bait (ATSB)—Barrier for control of vector and nuisance mosquitoes and its effect on non-target organisms in sub-tropical environments in Florida

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ABSTRACT

The efficacy of attractive toxic sugar baits (ATSB) with the active ingredient eugenol, an Environmental Protection Agency exempt compound, was evaluated against vector and nuisance mosquitoes in both laboratory and field studies. In the laboratory, eugenol combined in attractive sugar bait (ASB) solution provided high levels of mortality for *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles quadrimaculatus*. Field studies demonstrated significant control: >70% reduction for *Aedes atlanticus*, *Aedes. infirmatus*, and *Culex nigripalpus* and >50% reduction for *Anopheles crucians*, *Uranotaenia sapphirina*, *Culiseta melanura*, and *Culex erraticus* three weeks post ATSB application. Furthermore, non-target feeding of six insect orders, Hymenoptera, Lepidoptera, Coleoptera, Diptera, Hemiptera, and Orthoptera, was evaluated in the field after application of a dyed-ASB to flowering and non-flowering vegetation. ASB feeding (staining) was determined by dissecting the guts and searching for food dye with a dissecting microscope. The potential impact of ATSB on non-targets, applied on green non-flowering vegetation was low for all non-target groups (0.9%). However, application of the ASB to flowering vegetation resulted in significant staining of the non-target insect orders. This highlights the need for application guidelines to reduce non-target effects. No mortality was observed in laboratory studies with predatory non-targets, spiders, praying mantis, or ground beetles, after feeding for three days on mosquitoes engorged on ATSB. Overall, our laboratory and field studies support the use of eugenol as an active ingredient for controlling important vector and nuisance mosquitoes when used as an ATSB toxin. This is the first study demonstrating effective control of anophelines in non-arid environments which suggest that even in highly competitive sugar rich environments this method could be used for control of malaria in Latin American countries.

1. Introduction

Malaria was a major health issue in the United States until 1947 when The National Malaria Eradication Program tackled the mosquito-borne disease and eliminated malaria in the US by 1951 (Centers for Disease Control and Prevention (CDC), 2010). Currently diseases such as West Nile virus (WNV), eastern equine encephalitis virus (EEE), and dengue virus are public health concerns and major focuses of mosquito control programs in the US. In Latin America, however, approximately 170 million people live in malaria endemic areas where 1–1.2 million clinical malaria cases occur every year (Guerra et al., 2010; World Health Organization (WHO), 2009). Control has fallen short due to parasite resistance to anti-malarial drugs (Corredor et al., 2010; Feachem et al., 2009; World Health Organization (WHO), 2005), mosquito resistance to DDT and other insecticides, economical constraints, and unclear malaria control policies (Roberts and Andre, 1994; World Health Organization (WHO), 1998). One new method that has proven to

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14. ABSTRACT

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be successful for anopheline control in arid environments is attractive toxic sugar baits (ATSB). ATSB field trials applied as a foliar or surface space spray has demonstrated effective control of important malaria vectors in arid and semi-arid environments (Müller and Schlein, 2006; Müller et al., 2008, 2010a,c; Beier et al., 2012). Nonetheless, control of anopheline mosquitoes using ATSB has not yet been demonstrated in more tropical, sub-tropical environments like Latin America or Florida, US.

ATSB methods work by using the drive of both males and females for daily sugar meals against itself (Yuval, 1992; Foster, 1995). As mosquitoes search for a sugar source the highly attractive combination of juices attracts the mosquitoes away from their natural sugar sources and mortality ensues after ingesting a low dose of insecticide that is contained in the bait. "Attract and kill" concepts such as this have been highly successful for decades in agriculture and pest control. Nevertheless, only within the past decade has the idea of attract and kill been evaluated for use in mosquito control with further development needed for successful integration into abatement programs (Xue et al., 2013).

Control of *Aedes albopictus* was demonstrated in a recent study in sub-tropical environments in the USA using a foliar ATSB application (Xue et al., 2006; Naranjo et al., 2013). With the success of this evaluation, understanding the impacts of ATSB on other important mosquito populations in sub-tropical environments is imperative for the implementation of ATSB in integrated vector management (IVM) programs including those that are aimed at dengue and malaria control. The objective of this study was to determine the efficacy of a large-scale barrier application of ATSB for control of important vector and nuisance mosquitoes in sub-tropical environments using the environmentally friendly active ingredient, eugenol. Additionally, we evaluated the potential impact of the new control method on non-target organisms in Florida.

2. Materials and methods

2.1. Mosquito laboratory evaluations

Laboratory studies were conducted with colonized *Aedes aegypti*, *Anopheles quadrimaculatus* and *Culex quinquefasciatus* reared following (the) methods of Gerberg et al. (1994). Adults were maintained on 10% sucrose solutions and maintained at 27–28 °C and 70–85% RH under a 14:10 (L:D) photoperiod until used in assays. Laboratory evaluations were conducted to verify palatability of the attractive sugar bait (ASB) solution and to compare the efficacy of eugenol against mosquitoes of different genera. Assays were conducted following Allan (2011) and consisted of placing 10 female mosquitoes (5–7 days old) of either *Ae. aegypti*, *An. quadrimaculatus* or *Cx. quinquefasciatus* into plastic cups (100 ml) covered with fabric screen. Sections of cotton dental wick (1 cm long) (Unipack Medical Corp., Commerce, CA) were saturated with solutions consisting of either 0.1%, 1.0%, or 10% eugenol in ASB (described below). Controls consisted of wicks saturated with the ASB solution, 10% sucrose solution, or starved controls (no water or bait solution). The latter were included in the event that eugenol reduced feeding. Cups were held in trays with moistened paper towels to provide humidity. Cups were not held in sealed trays with lids as preliminary studies indicated that mortality could occur in the presence of vapors of eugenol in closed spaces. Testing in this fashion allowed determination of mortality through ingestion of eugenol and not from vapors. To further verify that vapors were not causing mortality, 10 cups containing mosquitoes were provided with sucrose-treated wicks on the top of the screening and wicks containing 10% eugenol were positioned on a pin immediately above the screen but out of the reach of mosquitoes. Mortality was observed at 1, 4, and 24 h with mosquitoes considered dead if they were unable to stand and had no wing movement. For each dose, five assay cups of adult mosquitoes were tested and replicated on three different days. Additionally, food grade dye was added to some test solutions and mosquitoes dissected to verify ingestion.

2.2. Study site

The study was conducted at the St. Johns Golf and Country Club (SJGCC; 29.802016, −81.382586), Elkton, FL encompassing 202 ha. The SJGCC, with a community of over three hundred family homes, is surrounded by pine forests and wetlands. The open parkland of the golf course is a mixture of private gardens, often with copious ornamental plants, wetland habitats, and numerous large ponds. The experimental site was between a pond and the pine forest while the untreated control site was between backyards and a pine forest (Fig. 1). Populations of *Anopheles crucians* and *Culex nigripalpus* at the study site can remain high during wet years.

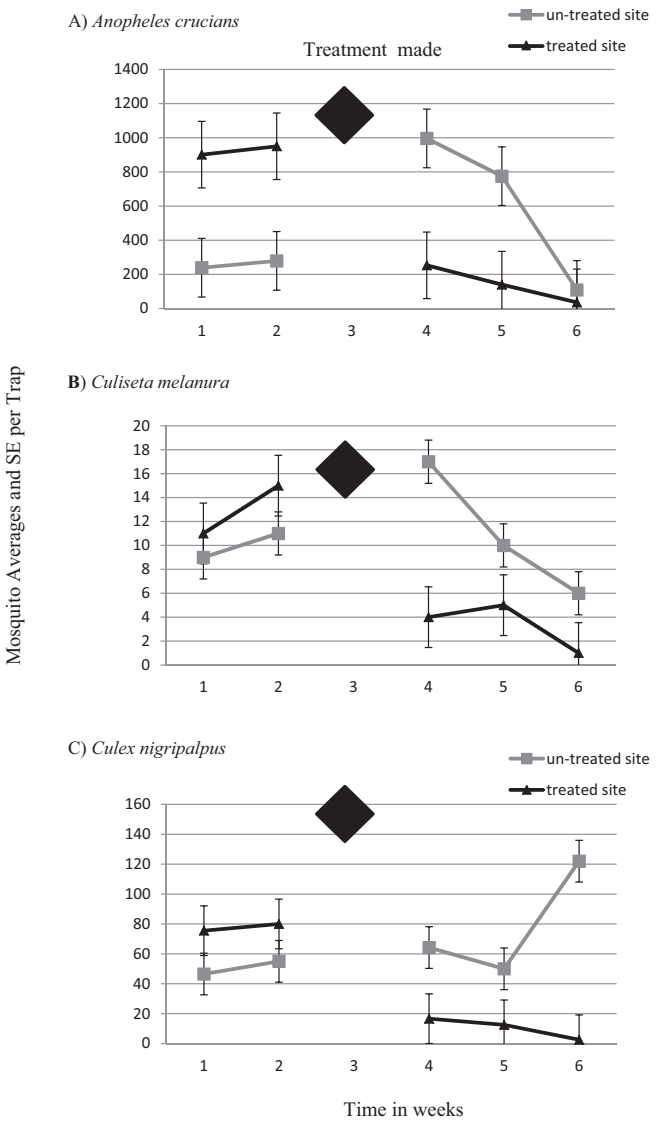

Fig. 1. Numbers (mean ± SE) (A) *Anopheles crucians*, (B) *Culiseta melanura* and (C) *Culex nigripalpus* captured by light traps at the untreated and treated sites pre and post attractive toxic sugar bait application.

2.3. Preparation of ATSB solutions

ASB for spraying was prepared from industrial grade attractive sugar bait concentrate (supplied by Westham Ltd, Tel Aviv, Israel) by diluting concentrate 1:4 in regular tap water and mixing 1:1 white unrefined sugar with tap water. ATSB was prepared in the same manner as ASB but included the addition of the 25b Environmental Protection Agency (EPA, 2013) minimum risk pesticides eugenol at 0.8% w/w%. Minimum risk pesticides are a special class of pesticides that are not subject to federal registration requirements because their ingredients, both active and inert, are demonstrably safe for the intended use.

For the non-target experiments no active ingredient was added. Due to the fact that prepared baits are typically invisible after applied to vegetation, we added (1:200) blue (blue food dye no. 1) or red (Azorubine food dye (Stern, Natanya, Israel) to stain the bait. Insects feeding on the bait can be identified by their color stained guts (Schlein and Müller, 2008).

2.4. Barrier application of ATSB

A treatment site of 1 km was selected for ATSB application. Application efficacy was evaluated with six CDC light traps (John W. Hock, Gainesville, FL) baited with dry ice. The traps surrounding the ATSB barrier application were separated by 100 m. The control site was located 800 m from the treatment site. Control mosquito populations were monitored in the same manner as described for the treatment site.

One ATSB application was made with a flo jet pump calibrated at 3.7 LPM and a 40° flat fan nozzle. ATV mounted spray equipment was used for spraying. The driver was moving 8 km/h along the forest edge while a technician sprayed the vegetation, moving the nozzle up and downward. A total of 113 l was applied to non-flowering vegetation 1 to 2 m deep and 1 to 2 m high. Approximately, one third of the golf course was treated with the ATSB barrier application. The field trial was conducted over a period of 28 days from November 3rd to the 30th, 2012. During this period, collected mosquitoes were counted and identified to species. The percent reduction between the control and treatment site was calculated using the formula $((P+C)-T/(P+C)$ where P stands for populations before treatment, C stands for populations at the control site, and T stands for populations at the treatment site (Mulla et al., 1971).

2.5. Non-target evaluation

Non-target field studies evaluating insect feeding from the selected six orders on vegetation treated with ASB was conducted by dissecting and examining guts for food dye under a dissecting microscope. The insect orders included: Hymenoptera (with focus on Aculeata including honey bee (*Apis mellifera*), wild bees and wasps), Lepidoptera (including Rhopalocera, all families of Macroheterocera and Microlepidoptera), Coleoptera (with focus on Carabidae, Tenebrionidae, Scarabaeidae, Cerambycidae, Chrysomelidae), Diptera (Brachycera only), Hemiptera (including Cicadomorpha and Heteroptera) and Orthoptera (Caelifera and Ensifera).

One hectare, near the SJGCC, was treated with either the blue or red stained ASB solution using a backpack pressure sprayer (Pestro 2000 Backpacksprayer, B&G, GA). Treatments were made to 5% of the vegetation and latter were characterized as flowering or non-flowering green vegetation (Schlein and Müller, 2008). The food dye colored the guts of insects that fed on the bait for at least for 24 h. The percentage of stained insects after the first day of ASB application can therefore be seen as a potential maximal daily feeding/killing rate (Schlein and Müller, 2008).

We followed closely existing EPA guidelines by applying the test substance at the rate, frequency, and method specified on the label [EPA 712-C-017] (EPA, 2012a,b,c). The test conditions for conducting an actual field test should resemble the conditions likely to be encountered under actual use of the product. In the absence of specific EPA guidelines for non-target evaluations, we designed the non-target experiments coming as close as possible to use of the product under field conditions.

Non-target insects were monitored the day and night immediately after the ASB application at the treated site with 50 yellow plates (yellow disposable plastic Plates 25 cm diameter filled with water and a drop of Triton-X as detergent), 4 malaise traps (2 and 6 m; Model 2875D, BioQuip, Rancho Dominguez, CA), two large UV-light traps (generator powered 250 ML light bulb mounted in front a white 2 × 5 m white linen sheet), six UV-tray traps (Müller et al., 2011), 50 pitfall traps (500 ml plastic cups buried to the rim in the ground, baited with 10 ml vinegar) (Leather, 2005), sweep-nets (BioQuip, Rancho Dominguez, CA) (two collectors), and entomological hand nets (BioQuip, Rancho Dominguez, CA) (two collectors) (see Müller et al., 2005; Müller et al., 2006 for more detailed description of sampling). Collected insects were stored at −20 °C in a freezer before being processed. Traps were kept at a distance of at least 5 m to treated patches of vegetation while manual collecting was done at random over the treatment site.

Because of the large number of non-targets that were collected, aliquots from each collecting method were used to determine the percentage of stained insects. Again, due to the volume of the collections, morpho-species, species that are distinct based on morphological characteristics, were identified instead of identifying each specimen to species level.

Experiments with predators were conducted under semi-field conditions in St. Augustine, nearby the experimental site. Predatory invertebrates that included (Araneae), praying mantis (Mantoidea), and ground beetles (Carabidae) were collected in the field and transferred individually to 20 × 20 × 20 cm plastic trays [with a layer of 1 cm of local sandy soil and some dry leaves]. The trays were closed with gauze and kept on a table in the shade of a large sun umbrella. The predators were fed in the trays with micro forceps with ATSB engorged, wingless but living mosquitoes. Feeding was verified by visual observation and resulting mortality was evaluated twice a day at 12-h intervals for three consecutive days.

2.6. Statistical analysis

Laboratory data did not fit a normal distribution as determined by a Shapiro–Wilk test and at each time interval and for each species, analysis was conducted using the Kruskal–Wallis. Means within each time interval for each species were separated using Dunn's pairwise multiple comparison test ($P < 0.05$).

A generalized linear models analysis was used to analyze the count data, which had a Poisson distribution. Marked overdispersion of the data was noted; therefore, we used a negative binomial analysis that incorporated a scale parameter into a Poisson regression to control for the overdispersion. The model for the ATSB barrier analysis included treatment group (control/treatment), day, and the treatment by day interaction. Separate analyses were run for each species. Back-transformed means and standard errors are shown graphically. The model for the non-target analysis included species, vegetation type (flowering/non-flowering), and the interaction of species and vegetation. The total number of insects inspected for each species was used as an offset. Back-transformed percents and standard errors are presented. The P-value of planned comparisons between vegetation types is also presented for each species. The 0.05 significance level was used to determine

Table 1
Percent mortality (SE) of adult female mosquitoes in laboratory bioassays after 1,4, and 24 exposure to cotton wicks saturated with solutions of ASB or 10% sucrose (controls) or eugenol (0.1% 1%, 10%) in ASB. Additional controls consisted of starved mosquitoes.

	Aedes aegypti			*Anopheles quadrimaculatus*			*Culex quinquefasciatus*		
	1	4	24	1	4	24	1	4	24
10% Eugenol/ASB	60.0 ((4.5)a	90.0 ((3.4)a	100.0 ((0.0)a	1.8 ((1.3)a	6.8 ((2.9)a	46.2 ((7.1)b	68.0 ((5.2)a	100 ((0.0)a	100 ((0.0)a
1% Eugenol/ASB	39.3 ((11.6)b	37.3 ((12.3)b	98.0 ((1.1)a	10.6 ((3.7)a	73.1 ((7.9)a	87.5 ((6.3)a	32.0 ((7.0)b	35.3 ((11.9)b	98.0 ((1.4)a
0.1% Eugenol/ASB	10.7 ((4.3)b	11.3 ((3.1)c	97.3 ((1.8)a	8.7 ((2.8)a	21.8 ((6.3)b	38.7 ((6.3)b	9.6 ((3.7)c	11.3 ((3.1)c	100 ((0.0)a
ASB control	2.7 ((1.5)b	4.0 ((4.0)c	8.7 ((3.1)b	0.0 ((0.0)a	0.0 ((0.0)a	1.2 ((0.8)c	2.0 ((1.0)c	4.0 ((1.3)c	7.3 ((2.5)b
Sucrose control	0.0 ((0.0)b	(2.0)c	2.0 ((1.4)b	0.0 ((0.0)a	0.0 ((0.0)a	0.0 ((0.0)c	0.0 ((0.0)c	0.0 ((0.0)c	3.3 ((1.6)b
Starved	0.0 ((0.0)b	9.0 ((4.3)c	3.4 ((5.3)b	0.0 ((0.0)a	0.0 ((0.0)a	10.1 ((3.8)c	2.7 ((1.2)c	1.3 ((0.9)c	17.3 ((41)b
P[a]	<0.001	<0.001	<0.001	0.049	<0.001	<0.001	<0.001	<0.001	<0.001

Means within each column followed by different letters are significantly different (Dunn's test).
[a] Comparison by Kruskal–Wallis ANOVA on ranks at each time interval ($P < 0.05$).

statistical significance. SAS 9.3 (SAS Institute, Inc., Cary, NC) was used for all analyses.

3. Results

3.1. Laboratory evaluation

Eugenol combined in ASB solution provided high levels of mortality in all species tested (Table 1). Mortality was low when mosquitoes were provided with the 10% sucrose solution or ASB solution. Mosquitoes that were not provided with water or sucrose also had low levels of mortality. At 1 h, mortality was greatest and higher than controls after exposure to 1% and 10% eugenol for *Ae. aegypti* and *Cx. quinquefasciatus* but not for *An. quadrimaculatus*. By 4 h, greatest mortality for *Ae. aegypti* and *Cx. quinquefasciatus* was after exposure to 1% and 10% eugenol solution, however, for *An. quadrimaculatus* mortality was greatest with 1% eugenol solutions. By 24 h, almost complete mortality was obtained after exposure to 0.1%, 1%, and 10% eugenol solutions for both *Ae. aegypti* and *Cx. quinquefasciatus*. Highest mortality of *An. quadrimaculatus* at 24 h was obtained with 1% eugenol and was two-fold greater than with 0.1% or 10% eugenol. The reduced mortality by *An. quadrimaculatus* at 10% eugenol likely is a result of feeding repellency at this concentration as indicated by the lack of dye detected in many of the living or dead mosquitoes at this dose.

3.2. ATSB barrier application

A total of 23,980 mosquitoes in five genera were collected during this study. A single application of ATSB reduced mosquito densities of all six species collected (Table 2). Overall mosquito densities from the pre-treatment period decreased 8.4-fold (days 1–7) compared to the 2.4-fold increase (days 8–20) in the natural population at the control site that did not receive ATSB treatment. At the control site mosquito densities averaged per trap 296.2 ± 12.4 before and 715.7 ± 47.2 after the ATSB application was made at the treatment site. At the treatment site mosquito densities averaged per trap 1065.3 ± 88.1 before and 161.7 ± 20.2 after ATSB application at the treatment site.

Table 2
Percent reduction of the mosquitoes collected using CDC light traps in the control and treatment sites before and after eugenol laced attractive toxic sugar bait barrier application.

Treatment	Percent reduction $((P+C)-T/(P+C))$
Aedes atlanticus	89 (278/311)
Aedes infirmatus	94 (101/107)
Anopheles crucians	62 (4264/6841)
Culiseta melanura	55 (66/121)
Culex erraticus	57 (54/95)
Culex nigripalpus	70 (446/636)
Uranotaenia sapphirina	69 (40/58)

The most abundant mosquito collected was *An. crucians* which was significantly reduced compared to the control site ($F = 10.48$, $df_{1,2} = 1, 40$, $P = 0.002$) and at all weeks post-ATSB application ($F = 32.45$, $df_{1,2} = 3, 40$, $P < 0.001$) (Fig. 1). Densities of *An. crucians* from the pre-treatment period (days 1–7) decreased over 6.2-fold compared to over a 3.3-fold increase (days 8–20) in the natural population at the control site that did not receive ATSB treatment. At the control site densities of *An. crucians* averaged per trap 239.6 ± 57.3 before and 775.6 ± 184.5 after ATSB application at the treatment site. At the treatment site *An. crucians* averaged 901.6 ± 214.5 pre-treatment and 143.2 ± 34.3 post-ATSB treatment.

For, another important vector, *Cx. nigripalpus*, populations were decreased significantly compared to the control ($F = 5.64$, $df_{1,2} = 1, 40$, $P = 0.022$) (Fig. 1). There were significant interactions between treatment and week ($F = 17.7$, $df_{1,2} = 3, 40$, $P < 0.001$) with all three weeks demonstrating a decrease in population compared to the control site. Densities of *Cx. nigripalpus* from the pre-treatment period (1–7 days) decreased over 7.2-fold (8–20 days) compared to over a one-fold increase at the control site that did not receive ATSB treatment. At the control site densities of *Cx. nigripalpus* averaged per trap 30.5 ± 2.9 and 40.9 ± 8.8 after ATSB application at the treatment site. At the treatment site *Cx. nigripalpus* averaged 75.5 ± 17.6 pre-treatment and 10.5 ± 0.5 post-ATSB treatment.

Culiseta melanura populations were decreased significantly compared to the control site ($F = 19.76$, $df_{1,2} = 1, 40$, $P < 0.001$) (Fig. 1). There was a significant reduction of *Cs. melanura* populations up to two weeks post ATSB application compared to the control site ($F = 4.59$, $df_{1,2} = 3, 40$, $P = 0.008$). Densities of *Cs. melanura* from the pre-treatment period (8–20 days) decreased over two-fold compared to less than one-fold natural decrease at the control site that did not receive ATSB treatment. At the control site densities per trap of *Cs. melanura* averaged 18.3 ± 5.4 before and 8.9 ± 3.2 after ATSB application at the treatment site. At the treatment site *Cs. melanura* averaged 26.6 ± 7.3 pre-treatment and 7.3 ± 1.9 post-ATSB treatment.

Aedes atlanticus populations were decreased significantly compared to the control site ($F = 5.4$ $df_{1,2} = 1, 40$, $P = 0.025$). There was a significant reduction of *Ae. atlanticus* populations up to three weeks post ATSB application compared to the control site ($F = 5.13$, $df_{1,2} = 3, 40$, $P = 0.004$). Densities of *Ae. atlanticus* from the pre-treatment period (1–7 days) decreased over 6.9-fold (8–20 days) compared to over a two-fold increase at the control site that did not receive ATSB treatment. At the control site densities per trap of *Ae. atlanticus* averaged 13.8 ± 3.5 before and 16.8 ± 4.7 after ATSB application at the treatment site. At the treatment site *Ae. atlanticus* averaged 38.1 ± 6.5 pre-treatment and 1.8 ± 0.5 post-ATSB treatment.

Aedes infirmatus populations were decreased significantly compared to the control site ($F = 4.4$ $df_{1,2} = 1, 40$, $P = 0.03$). There was a significant reduction of *Ae. infirmatus* populations up to three weeks post ATSB application compared to the control site ($F = 18.2$, $df_{1,2} = 3, 40$, $P < 0.0001$). Densities of *Ae. infirmatus* from the pre-treatment period (1–7 days) decreased over 30-fold compared to a 1-fold natural increase at the control site that did not receive ATSB treatment. At the control site densities per trap of *Ae. infirmatus* averaged 1.6 ± 0.9 before and 8.8 ± 5.8 after ATSB application at the treatment site. At the treatment site *Ae. infirmatus* averaged 16.1 ± 2.4 pre-treatment and 0.3 ± 0.2 post-ATSB treatment.

Culex erraticus populations were decreased significantly compared to the control site ($F = 5.6$ $df_{1,2} = 1, 40$, $P = 0.022$). There was a significant reduction of *Cx. erraticus* populations up to three weeks post ATSB application compared to the control site ($F = 17.7$, $df_{1,2} = 3, 40$, $P < 0.0001$). Densities of *Cx. erraticus* from the pre-treatment period (1–7 days) decreased over 2.2-fold compared to a 13.8-fold natural increase at the control site that did not receive ATSB treatment. At the control site densities per trap of *Cx. erraticus* averaged 2.0 ± 0.5 before and 8.7 ± 4.5 after ATSB application at the treatment site. At the treatment site *Cx. erraticus* averaged 12.8 ± 3.6 pre-treatment and 2.2 ± 0.8 post-ATSB treatment.

Uranotaenia sapphirina populations were decreased significantly compared to the control site ($F = 7.9$ $df_{1,2} = 1, 40$, $P = 0.020$). There was a significant reduction of *Ur. sapphirina* populations up to three weeks post ATSB application compared to the control site ($F = 6.27$, $df_{1,2} = 3, 40$, $P = 0.001$). Densities of *Ur. sapphirina* from the pre-treatment period (1–7 days) decreased over 2.2-fold compared to a 13.8-fold natural increase at the control site that did not receive ATSB treatment. At the control site densities per trap of *Ur. sapphirina* were 0 before and 8.3 ± 2.5 after ATSB application at the treatment site. At the treatment site *Ur. sapphirina* averaged 9.6 ± 1.5 pre-treatment and 1.0 ± 0.2 post-ATSB treatment.

3.3. Non-target evaluation

There were significant differences between the staining of mosquitoes and non-targets for both sugar rich and sugar poor sites (Table 3). The potential impact on non-target insects of ATSB applied on flowering vegetation was high for higher Diptera, Hymenoptera, and Lepidoptera whereas the impact on the insect orders Orthoptera, Coleoptera, and Hemiptera was low. Over 10% of the non-targets were stained in the flowering vegetation site. Overall the impact on non-target insects of ATSB applied on green non-flowering vegetation was low for all non-target orders as only 0.9% of the individual insects were stained with the dye from the ASB solutions. There were no significant differences between the staining of mosquitoes collected in flowering vegetation site (241/1000) or the non-flowering site (270/1000) during the non-target evaluation.

No mortality was observed in predatory invertebrates, net spiders (0/20), praying mantis (0/10), and ground beetles (0/20) after feeding for three days on mosquitoes engorged on ATSB applied to vegetation. There was no control mortality of the untreated (starved) control spiders (0/20), praying mantis (0/10), and ground beetles (0/20).

4. Discussion

Laboratory experiments demonstrated that the EPA exempt active ingredient eugenol was effective at controlling vector mosquitoes when applied in sugar solution. Our field tests corroborated the laboratory study when ATSB-eugenol application resulted in control of important vector and nuisance populations for three weeks post-application. Previous studies have evaluated eugenol as a repellent (Revay et al., 2013; Hao et al., 2008) and biolarvicide (Waliwitiya et al., 2008), however, this is the first study demonstrating eugenol as an effective stomach poison against mosquitoes. The mode of action is not clear but mortality is significant in mosquitoes after ingesting the 0.8% eugenol sugar bait. Interestingly, in our laboratory and field studies, eugenol used at low concentrations had no repellent effect on mosquitoes. The results presented here, with the addition of eugenol as the toxin, support previous field studies done in Israel (Müller et al., 2008), Mali (Müller et al., 2010a,c), the US (Xue et al., 2006; Naranjo et al., 2013) and Morocco (Khallaayoune et al., 2013) where mosquito populations were controlled after a spinosad, boric acid, and dinotefuran ATSB application, respectively. The addition of the industrial grade ASB concentrate increased the residual time of the ATSB application by three weeks compared to the previous study done by Xue et al. (2006) and Naranjo et al. (2013) which only found control for two weeks and one week, post-ATSB application, respectively. This increase in residual time is important for sustainability and incorporation into IVM strategies. Furthermore, this study identifies an additional active ingredient that can be used in combination with the attractive sugar baits for successful mosquito control highlighting the versatility of this method.

It is important to note that the ATSB method had significant impacts on important vector species involved in WNV and EEE transmission in FL. The WNV vector *Cx. nigripalpus* can be difficult to control because this species behavior is very dependent upon humidity. Humidity stimulates this species to take flight and allows mosquitoes to enter and host seek in habitats that are inhospitable when dry (Shaman et al., 2002). In periods of severe drought, *Cx. nigripalpus* will avoid areas such as open, sparsely vegetated, temperate and subtropical marshes, woodlands, fields, urban areas, and rural housing developments where they would normally be found residing (Day and Shaman, 2008). Until conditions are favorable, this species tends to rest and host-seek in heavily vegetated habitats increasing bird-mosquito contact and ultimately the number of WNV infected birds. Thus, targeting the heavily vegetative areas where this species is resting would likely decrease WNV amplification and human vector contact since the mosquitoes could be targeted before the favorable conditions to take flight are met.

The ATSB application method also works in the same manner for control of the important EEE vector, *Cs. melanura*. This species does not frequently feed on humans but is considered the enzoonotic reservoir for EEE. Targeting this species development and resting habitats using the ATSB method would interrupt the EEE enzoonotic cycle and decrease human and veterinary risk of contracting EEE.

Though *An. crucians* is not considered to be a competent vector of malaria in the US or Latin America our field studies demonstrate that sub-tropical anopheline species can be controlled with the ATSB method. More importantly our laboratory studies demonstrate control of a competent North American malaria vector, *An. quadrimaculatus* using eugenol-baited ATSB. Previous studies have demonstrated decimation of important malaria vectors in arid and semi-arid environments (Müller and Schlein, 2006; Müller et al., 2008, 2010a,c; Beier et al., 2012). This is the first report of anopheline control in non-arid environments. The abundance and diversity of flowering vegetation in arid and semi-arid environments are very spatially and temporally mediated. During certain times of the year in arid and semi-arid environments, sugar sources may not be readily available for mosquitoes. In more tropical and semi-tropical environments flowering vegetation is almost always abundant. The ATSB method success in arid and semi-arid environments has been attributed to this lack of readily available sugar sources. However, we demonstrate that even in highly competitive sugar rich environments many vector and nuisance species can be successfully controlled. These findings suggest that malaria control in tropical

Table 3
Percent attractive sugar bait stained ±SE of each insect order collected after application of attractive sugar bait application to flowering vegetation and non-flowering vegetation.

Species	Flowering vegetation		Non-flowering vegetation		Flowering vs. Non-flowering vegetation
	%[a]	±SE	%[a]	±SE	P^{b}
Mosquitoes	24.10	14.89	27.00	16.68	0.898
Coleoptera	5.18a	1.40	0.69a	0.23	<0.001
Diptera[*]	17.85	11.01	1.45a	0.93	0.009
Hemiptera	3.21a	1.57	0.27a	0.23	0.017
Hymenoptera	15.49	5.55	0.85a	0.35	<0.001
Lepidoptera	6.71	1.74	0.75a	0.24	<0.001
Orthoptera	1.25a	0.95	0.50a	0.47	0.454

[a] Columns with different letters indicate significant differences in staining rate compared to the control group (mosquitoes).
[b] Comparison of stained orders of flowering vegetation vs. non-flowering vegetation
[*] Without mosquitoes.

environments like Latin America, where new control methods are urgently needed, may be feasible.

This study demonstrates that ATSB applications in sub-tropical environments would have very little impact on non-target arthropods. When the ASB was applied to flowering vegetation, non-target populations were significantly stained suggesting that some non-target populations would be unable to recover. However, when the ASB was applied to non-flowering vegetation non-target insect populations were not attracted to the baits and did not feed on them. Most likely, the ASB-treated green vegetation did not provide a visual attractive target for pollinators providing an explanation for our findings. In order to stand out from the predominant green colors of leaves and stems plants have flowers and fruits that vary in color. These colors create optical signals that are used to attract insect pollinators (Lee, 2007). As a result, ATSB applications, as long as they are applied to green, non-flowering vegetation would have little attractancy to non-target pollinators and avoid any potential unacceptable negative impact as demonstrated in this study. The development of bait stations made with protective grids allowing only small biting flies to feed while excluding other insects like honey bees could further enhance the ATSB strategy to reduce non-target effects. These studies provide essential non-target data that is needed for the development of clear guidelines for appropriate use and adaptation into IVM programs of the new ATSB control method.

In the present study negative impacts of the ATSB method on predatory arthropods were not observed even though the predators were forced to feed on the contaminated pray which will never correspond to field conditions. Our findings are consistent with previous studies where there was no significant mortality associated with ATSB-fed predators (Khallaayoune et al., 2013). Since ATSB has to be ingested and is not a contact poison, predatory insects that generally do not feed on plant material have a low probability of being affected by ATSB applications.

While non-target arthropods were not attracted to nor feeding on the ASB application to non-flowering vegetation, mosquitoes had a high level of staining indicating ingestion of the bait at levels likely sufficient for control. ATSB and boric acid sugar baits sprayed on flowers and non-flowering vegetation resulted in similar effective control of adult mosquitoes (Müller et al., 2010b; Xue et al., 2011). Mosquitoes appear to be guided more by scent than optical targets when sugar seeking unlike in host-seeking which is influenced by color and shape of the object (Allan et al., 1987). Müller et al. (2010c) demonstrated effective control in a storm drain systems by comparing the numbers of mosquitoes captured feeding on the ASB solution to the numbers captured after an ATSB application. Qualls et al. (2012) demonstrated a high level of staining from feeding on ASB (90%) by mosquitoes emerging from cisterns and wells. Based on the demonstrated level of staining, the ATSB application would be successful in controlling mosquitoes. Other studies incorporating a dyed ASB control have demonstrated in most cases >50% staining rate while achieving at least that percentage of control in the ATSB treatment sites (Müller and Schlein, 2006, 2008; Schlein and Müller, 2008; Müller et al., 2008, 2010a,b; Beier et al., 2012).

This study demonstrates that this novel control strategy applied to non-flowering vegetation is effective and has low non-target impact. Moreover, the EPA exempt active eugenol combined with the industrial quality bait provided control of important vector mosquito species for at least three weeks post-application in sub-tropical environments. The low non-target impact and increased sustainability of the ATSB method evaluated in this report further supports this as an IVM strategy that can be incorporated into vector control programs. Furthermore, the success of the barrier ATSB application in the sub-tropical environment suggest that this method could be used to combat malaria and other important mosquito-borne diseases that are a major public health burden in Latin America.

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